Attachment A:
Pages 7-12 of the original specification with mark-up.

BRIEF DESCRIPTION OF THE DRAWINGS

The invention will now be described, by way of example only and with reference to the following embodiments:

Figure 1 shows the nucleotide and deduced amino acid sequence of carp NGF and NT-7.

(A) The basic motif RVRR (double underlined) was the putative proteolytic cleavage site, followed by an open reading frame of 133 amino acids that constituted the mature protein (starting with the lysine residue as indicated by the arrowhead). Exact oligonucleotides at two conserved regions of *Xiphophorus* NT-6 (corresponded to nucleotide 51 to 68 and 374 to 390 of NT-7) were used to perform the initial PCR from carp genomic DNA that generated the partial sequence of NT-7. The conserved cysteine residues in the mature region, as well as the conserved sequence nearby, were underlined. There is a characteristic insertion of 15 amino acids between the second and third cysteine residues (bolded). Note the entire pre-pro region that contained the putative ATG start codon 141, 195, 208, and 283, with changes at the Ile^{70} and Glu^{95} to Val and Lys, respectively.

(B) Partial nucleotide sequence and the deduced amino acid sequence of carp NGF. A 177-bp fragment corresponded to the partial sequence between

the second and the fifth conserved cysteine residues of carp NGF. The underlined sequence represented positions of the degenerate primers.

Figure 2 shows the alignment of carp NGF and NT-7 with other neurotrophins.

(A) Alignment of NGF and NT-7 with other known neurotrophins from lower vertebrates (Xipma represented *Xiphophorus maculatus*; *Xen* represented *Xenopus laevis*). Amino acids identical to that of NT-7 were represented by dots, while gaps were indicated by dash. Conserved regions among all the neurotrophins were underlined. The 15-amino-acid insertion (double underlined) was characteristic to this novel neurotrophin. The mature region of NT-7 shared 66% amino acid identity to Xipma NGF and NT-6 (without taking into account the insertion).

(B) Alignment of carp NGF and NT-7 with NGF from various animals. Only partial sequence of salmon NGF had been reported. The amino acid residues that were conserved in all known NGF but different in NT-7 were bolded.

Figure 3 shows a Southern blot analysis of *Hind*III-cut *Xiphophorus* genomic DNA. The digested DNA was hybridised with cDNA of *Xiphophorus* NGF, NT-6, or carp NT-7 under low stringency. Numbers on the left indicated the sizes of the *Hind*III-digested lambda marker DNA (in kilobases). Distinct bands of ~8 and ~3, and ~2 kb hybridised with *Xiphophorus* NGF, NT-6, and carp NT-7 respectively.

Figure 4 shows a Northern blot analysis of the spatial expression of NT-7 and NGF in adult fish.

(A) Spatial expression of NT-7 in adult carp was examined. A single transcript of ~1.1 kb was detected in skin and heart. Weak expression was also found in brain and intestine.

(B) Spatial expression of NGF in *Xiphophorus*. A transcript of ~3.6 kb was detected predominantly in eye and gill, while expression in skin was much weaker, in both A and B, the corresponding ethidium bromide-stained gel indicated similar amount of RNA loaded in each lane.

Figure 5 is an RT-PCT showing the presence of the 15 amino-acid insertion in the transcript of NT-7.

A fragment of 304 bp (indicated by upper arrowhead), which corresponded to the presence of the insertion, was produced after amplification from brain, heart, intestine, and skin cDNA by a pair of primers flanking the insertion (nucleotide 69 to 85 and 358 to 373 in Figure 1). The RNA was replaced by DEPC-H_2O in the no-RNA control, while the same concentration of RNA but without reverse transcriptase was used in the no-RT control. NT-7 and NT-7(D15) represented PCR products amplified by the same primers from the expression plasmids NT-7 and NT-7(D15) which lacked the 15-amino-acid insertion (indicated by lower arrowhead), respectively. These served as the size standards to demonstrate that the insertion was present in the transcripts from the various tissues.

Figure 6 shows the biological activities of the various fish neurotrophins. Photomicrographs showing E8 chick DRG after 1 day treatment with COS cell supernatant from mock-transfected cells (A), or from cells transfected with *Xiphophorus* NGF (B), NT-6 (C), NT-6(D22), (D), NT-7 (E), and NT-7(D15) (F). At least five DRGs were placed in each 35-mm dish coated with 1 ug/ml poly-o-lysine and treated with 1:5 diluted COS cell supernatant. The DRG were fixed and stained by anti-neurofilament 160-KDa antibody. Extensive neurite outgrowth was observed from DRG treated with *Xiphophorus* NGF, NT-7, or NT-7(D15) but not NT-6 and NT-6(D22). After pre-pro exchange with *Xiphophorus* NGF, NT-6 and NT-6(D22) were still unable to induce neurite outgrowth of DRG explant (data not shown). Magnification x60.

Figure 7 shows the survival of dissociated DRG neurons in response to the neurotrophins. Dissociated DRG neurons from E8 chick were planted onto 35-mm poly-D-lysine-coated plates, and the number of surviving neurons were counted after 1-day treatment with the COS cell supernatant (diluted 6:5 by medium). *Xiphophorus* NGF, NT-7, and NT-7(D15) were able to support survival of the neurons (50-60%) while less than 20% of the neurons survived in response to NT-6, NT-6*, NT-6(D22), or NT-6*(D22), which was similar to that of mock-transfected cells. NT-6* represented NT-6 with pre-pro region of NGF. The error bars represented SEM, n = 6.

Figure 8 shows phosphorylation of chick Trk receptors by the neurotrophins

(A) Chick TrkA was transiently transfected into 293 fibroblasts and assayed for phosphorylation by the COS cell supernatant (undiluted) that contained the

various fish neurotrophins. Similar observation was obtained using 6:5 diluted COS cell supernatant. The faster migrating band (about 96 kDa) represented contitutively phosphorylated precursor (Ibenez et al., 1993), and its signal could serve to equalise the amount of sample on each lane. All the fish neurotrophins examined, except NT-6, could weakly phosphorylate TrkA, but the extent of phosphorylation was considerably lower than that of *Xiphophorus* NGF. Similarly, NT-6(D22), but not NT-6, that contained NGF pre-pro region could induce weak TrkA phosphorylation (data not shown).

(B) Chick fibroblasts that were stably transfected with chick TrkB were used to test the ability of the neurotrophins to phosphorylate TrkB. Only BDNF (50 ng/ml), which served as the positive control, could phosphorylate the receptor.

(C) Phosphorylation of TrkC by the neurotrophins was tested by chick fibroblasts stably transfected with chick TrkC. All the fish neurotrophins were unable to phosphorylate the receptor, except NT-3 (50 ng/ml) which acted as the positive control (since only one-third of the positive control was loaded, the lower two bands that were present in the other samples were not visible).

Figure 9 shows neurite outgrowth of DRG in response to Fc-tagged neurotrophins. COS cell supernatant that contained equal amounts of the Fc-tagged NGF (B) or NT-6(D22), as determined by ELISA, was added to E8 chick DRG. Only NGF-Fe promoted the neurite outgrowth, while the activity of NT-6(D22)-Fe was similar to mock-transfected COS cell supernatant (A). The

activity of NT-6-Fc was also similar to the mock (data not shown).

Magnification: ~~x60~~.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to nucleic acid sequences encoding neurotrophin 7 (NT-7) as well as NT-7 protein, peptide fragments and derivatives produced using these nucleic acid sequences. In addition the invention relates to pharmaceutical compositions and therapeutic uses of NT-7.

The invention permits a comparison of the nucleic acid sequences of NT-7 and other neurotrophins.

MATERIALS AND METHODS

1 MOLECULAR CLONING OF XIPHOPHORUS NGF, NT-6, CARP NGF AND NT-7

In an attempt to clone the carp NT-6, two exact oligonucleotides representing the amino acid sequence YSVCDS (GTACTCTGTGTGTGACAG) SEQ ID NO: 3 & 4 and INAACV(CACACATGCAGCGTTGA) SEQ ID NO. 5 & 6, which corresponded to two conserved regions of NT-6, were designed ~~(Figure 1)~~. Carp genomic DNA (0.6 ug) was used in the polymerase chain reaction (PCR) as template. One-tenth of the reaction was further amplified by the same set of primers in a second PCR. The fragment was then gel purified and the ends were blunted by Klenow (Amersham, UK) before being ligated to *Sma*I-cut pBluescript™ (Stratagene, CA). After transformation into XL-1 blue, the plasmid was purified and double-